Neuron, Vol. 2, 1547–1558, June, 1989, Copyright © 1989 by Cell Press

Glutamate Toxicity in a Neuronal Cell Line Involves Inhibition of Cystine Transport Leading to Oxidative Stress

Timothy H. Murphy,*† Masaomi Miyamoto,‡ Antonio Sastre,† Ronald L. Schnaar,*‡ and Joseph T. Coyle*‡§
* Department of Pharmacology
† Department of Physiology
‡ Department of Neuroscience
§ Department of Psychiatry
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Summary

Glutamate binds to both excitatory neurotransmitter binding sites and a Cl^--dependent, quisqualate- and cystine-inhibited transport site on brain neurons. The neuroblastoma-primary retina hybrid cells (N18-RE-105) are susceptible to glutamate-induced cytotoxicity. The Cl^--dependent transport site to which glutamate and quisqualate (but not kainate or NMDA) bind has a higher affinity for cystine than for glutamate. Lowering cystine concentrations in the cell culture medium results in cytotoxicity similar to that induced by glutamate addition in its morphology, kinetics, and Ca^{2+} dependence. Glutamate-induced cytotoxicity is directly proportional to its ability to inhibit cystine uptake. Exposure to glutamate (or lowered cystine) causes a decrease in glutathione levels and an accumulation of intracellular peroxides. Like N18-RE-105 cells, primary rat hippocampal neurons (but not glia) in culture degenerate in medium with lowered cystine concentration. Thus, glutamate-induced cytotoxicity in N18-RE-105 cells is due to inhibition of cystine uptake, resulting in lowered glutathione levels leading to oxidative stress and cell death.

Introduction

We have studied L-glutamate (Glu) binding and toxicity using the neuronal hybridoma cell line N18-RE-105 (primary rat embryonic retinal cell × mouse neuroblastoma cell). Incubation of N18-RE-105 cells with Glu or Glu receptor agonists of the quisqualate (Quis) class results in delayed (6–10 hr), Ca^{2+}-dependent cell death (Murphy et al., 1988a). Since Quis has been reported to open cation channels (Jahr and Stevens, 1987), we previously evaluated depolarization as a mechanism of neuronal cell death (Murphy et al., 1988b). Although electrophysiological studies demonstrated that N18-RE-105 cells were mildly depolarized by application of Glu or Quis, depolarization per se was not cytotoxic. To the contrary, depolarization by KCl, veratridine, or ouabain actually reversed the cytotoxic effect of exogenously added Glu (Murphy et al., 1988b). Similarly, toxicity as a consequence of depolarization was not always observed in cerebellar brain slices (Garthwaite et al., 1986), and significant discrepancies between the neurotoxic and excitatory potency of agonists at Glu receptors have been reported (Lehmann et al., 1985). Thus, depolarization could not account for the cytotoxicity of Quis receptor agonists in N18-RE-105 cultures, suggesting that another mechanism must mediate their toxicity in this neuronal cell line.

Recent studies now indicate that Quis may interact with neuronal sites other than those mediating Glu-induced neuronal depolarization. One such site is a Cl^--dependent Glu transporter enriched in brain membranes, which was originally thought to be a Cl^--dependent receptor (Roberts, 1974; Berry et al., 1988; Kessler et al., 1987; Zaczek et al., 1987a, 1987b). This Quis-sensitive transporter was shown to be concentrated in neurons, as evidenced by a marked decrease in its activity upon a selective neuronal lesion (Zaczek et al., 1987b). L-Cystine (cystine) inhibits Glu sequestration at this Quis-sensitive transporter (Berry et al., 1988; Kessler et al., 1987; Zaczek et al., 1987a). This carrier site may be a high affinity neuronal adaptation of an antiporter for cystine described in the periphery (Bannai, 1986; Bannai and Kitamura, 1980, 1981; Makowske and Christensen, 1982; Bannai and Ishii, 1988). The interaction of Glu and cystine at the peripheral carriers is competitive, with the affinity of Glu one-fifth that of cystine (Bannai and Kitamura, 1980).

Using the N18-RE-105 cell line, we now report the role of Cl^--dependent cystine uptake in Glu's cytotoxicity and the general role of cystine uptake in cell viability. We present evidence that a Cl^--dependent Glu uptake system in N18-RE-105 cells functions as a cystine/Glu antiport transporter and that Glu toxicity is due to inhibition of cystine uptake. Cellular deprivation of cystine ultimately leads to oxidative stress through a reduction in levels of cellular oxidized and reduced glutathione (GSH and GSSG), resulting in delayed cell death. In primary cultures of mixed hippocampal neurons and glia, neurons, but not glia, are as sensitive to low cystine toxicity as N18-RE-105 cells. Thus, Glu-induced inhibition of cystine transport may contribute to certain forms of delayed neuronal degeneration.

Results

Reduced Cystine Concentration and Cytotoxicity of N18-RE-105 Cultures

Exposure of N18-RE-105 cells to medium deficient in cystine resulted in marked cell death. Toxicity in low cystine medium was morphologically indistinguishable from Glu-induced cytotoxicity: cell bodies first swelled and then neurites displayed numerous surface blebs prior to cell lysis. Reducing extracellular cystine from 200 μM, that in normal Dulbecco's modified Eagle's medium (DMEM), to 100 μM caused measurable cytotoxicity after 24 hr; and up to 80% cell lysis occurred in 24 hr when cystine was reduced to less than 10 μM (Figure 1). The relationship between cystine concentration and cytotox-

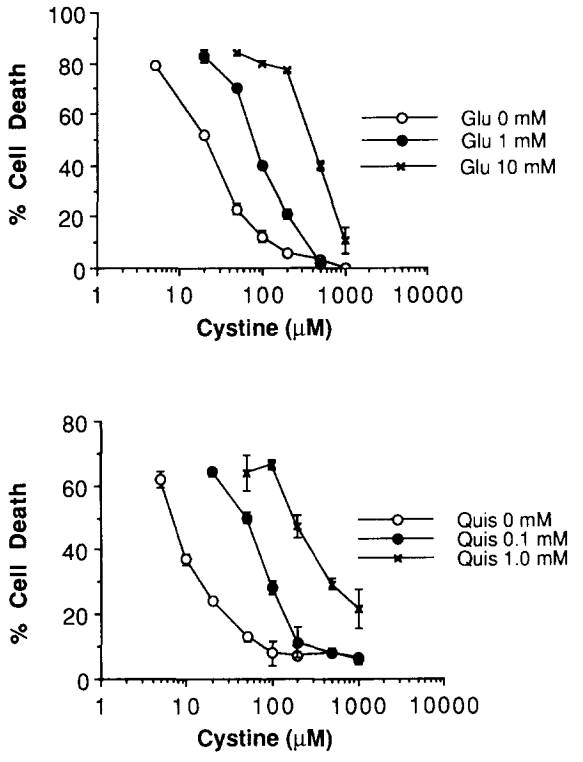

Figure 1. Toxicity of Low Cystine Culture Medium and the Effects of Glu and Quis

Media with various levels of cystine and/or Glu and Quis as indicated were prepared and incubated with N18-RE-105 cells for 24 hr, as described in Experimental Procedures. Cell death was quantified by measurement of cellular and released LDH. Values are the means ± SEM of a typical experiment performed in triplicate.

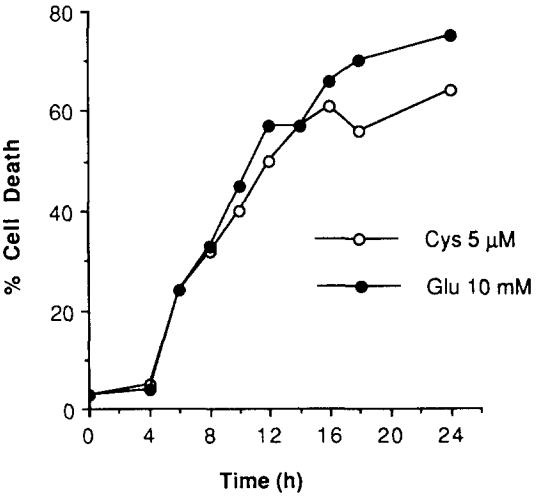

Figure 2. Time Course of Low Cystine and Glu Toxicity in N18-RE-105 Cultures

Cells were grown as described in Experimental Procedures. After the indicated time following switching to toxic medium, cell death was quantified by measurement of cellular and released LDH (see Experimental Procedures). Maximal cell death was 75% with 10 mM Glu and 64% with 5 μM cystine.

icity was analyzed by nonlinear regression and could be fit to the equation

$$\% \text{ death} = M - ((100 \cdot [cys])/([cys] + K))$$

in which M is the maximum percentage of dead cells and K is the concentration of cystine that induces 50% of maximal cell death, with K and M as free parameters. The average K for cell death was 11 ± 3 μM (n = 4 separate experiments with triplicate determinations). Data for cystine concentration versus cell death were also analyzed according to the Hill equation:

$$\% \text{ death} = M - ((100 \cdot [cys]^{n_H})/([cys]^{n_H} + K))$$

Fitted with K, n_H, and M as free parameters, the results suggest that a single noninteracting binding event (n_H = 1.06 ± 0.1, n = 4 separate triplicate determinations) could mediate low cystine-induced toxicity.

Effect of Glu on Low Cystine-Induced Cytotoxicity in N18-RE-105 Cultures

The above data suggested that lack of cystine kills cells and that Glu may be toxic due to its ability to block cystine uptake. In support of this hypothesis, Figure 1 demonstrates that addition of Glu to the medium results in cell death at a higher cystine concentration than observed in its absence. While half-maximal cell death in the absence of Glu occurred when the cystine concentration was lowered (from 200 μM) to 11 ± 3 μM (see above), in the presence of 1 mM Glu a cystine concentration lowered to 129 ± 27 μM was required for 50% cell loss (n = 3 experiments with triplicate determination; $P < 0.05$ compared with the absence of Glu). Addition of Glu (1–10 mM), or Quis (0.1–1.0 mM) resulted in shifts in the low cystine toxicity curves, which were consistent with competitive interactions of cystine/Glu or cystine/Quis at the same site (Figure 1).

Time Course of Low Cystine Toxicity in N18-RE-105 Cultures

Onset of cell lysis was not apparent until after 4–8 hr of exposure to low cystine (5.0 μM) medium, a time course nearly identical to that observed after the addition of 10 mM Glu to complete medium (Figure 2). The time to 50% of maximal cell loss was 8 hr after switching to low cystine medium and 9 hr after addition of Glu to complete medium. In a second experiment (data not shown), the time to 50% of maximal cell loss was 12 hr at 2.5 μM cystine exposure and 13 hr with 10 mM Glu exposure in complete medium.

Ca^{2+} Dependence of Low Cystine Toxicity in N18-RE-105 Cultures

Glu cytotoxicity in N18-RE-105 cells is prevented by incubation in medium deficient in Ca^{2+} (Murphy et al., 1988a). Similarly, toxicity due to reduced cystine medium was Ca^{2+}-dependent (Table 1). Reducing free Ca^{2+} levels to <10 μM completely blocked the cytotoxic effect of low cystine, although the Ca^{2+}-free condition was associated with a low rate of cell lysis (12% lactate dehydrogenase [LDH] release). As with Glu toxicity in

Table 1. Ca^{2+} Dependence of Low Cystine Toxicity

EGTA (mM)	Added Ca^{2+} (mM)	Free Ca^{2+} (μM)	Cystine (μM)	% Toxicity
0	1.8	ND	200	3 ± 1
0	1.8	ND	5	54 ± 1
0.5	0	<10	200	12 ± 4
0.5	0	<10	5	12 ± 4
0.5	1.8	1300	200	2 ± 1
0.5	1.8	1300	5	62 ± 4[a]

N18-RE-105 cells were grown for 24 hr as described in Experimental Procedures and then switched to the culture medium described. Cell death was determined by measurement of LDH release as described in Experimental Procedures. Results are the means ± SEM for 3 experiments performed in triplicate. ND, not determined.
[a] $P < 0.05$ (two-tailed paired t-test comparing cell death in 5 versus 200 μM cystine medium for each conditon).

N18-RE-105 cells (Murphy et al., 1988a), the inorganic Ca^{2+} channel blocker $CdCl_2$ (2.5 μM) was able to prevent completely the cytotoxicity due to low cystine medium (data not shown).

Cystine Uptake by N18-RE-105 Cells

Cystine uptake in N18-RE-105 was measured directly using L-[^{35}S]cystine. The K_t for cystine uptake was 20 ± 11 μM (Table 2), and the V_{max} was 52 ± 15 pmol/mg of protein per min. Specific uptake of 1 μM L-[^{35}S]cystine (specific activity 5 Ci/mmol) was linear for at least 75 min and resulted in accumulation of radiolabel at a rate of 8 pmol/mg of protein per min. After 75 min of uptake, the calculated concentration of radiolabel in cells ultimately exceeded that in the medium. Increasing the cystine concentration 1000-fold to 1 mM (reducing the specific activity to 5 mCi/mmol) reduced uptake of radiolabel to barely detectable levels, consistent with saturation of the carrier. Decreasing the specific activity by a factor of 1000 by the addition of unlabeled cystine (from 1 μM to 1 mM) after 20 min at the lower cystine concentration resulted in a cessation of radiolabel uptake, but no efflux of radiolabel, suggesting that cystine undergoes metabolic sequestration into a nonreleasable pool.

Uptake of L-[^{3}H]Glutamate in N18-RE-105 Cultures

The K_t for Glu uptake was 53 ± 16 μM (Table 2), and the V_{max} was 205 ± 52 pmol/mg of protein per min. Uptake of 10 μM L-[^{3}H]glutamate (specific activity 0.5 Ci/mmol) was biphasic. An initial component (1–20 min) of 81 pmol/mg of protein per min was followed by a slower component (20–75 min) of 31 pmol/mg of protein per min. The calculated concentration of radiolabel in cells ultimately exceeded that in the medium. Increase in the total Glu concentration to 10 mM (decrease of specific activity to 0.5 mCi/mmol) reduced time-dependent uptake of radiolabel to barely detectable levels. Efflux of >70% of 10 μM L-[^{3}H]glutamate accumulated after a 20 min incubation was detected upon dilution of specific activity 1000-fold by adding 10 mM unlabeled Glu, indicating that most of the accumulated L-[^{3}H]glutamate remains in an exchangeable pool. Subsequent uptake experiments were terminated after 20 min, which was the upper limit of the initial (rapid) component of uptake.

Table 2. Kinetic Constants for Cystine and Glu Uptake

Substrate	
	K_t (μM)
L-[^{35}S]Cystine	20 ± 11 (4)
L-[^{3}H]Glutamate	53 ± 16 (4)
	K_a L-[^{35}S]Cystine μM
L-Cystine	7.5 ± 1.6 (6)
L-Glutamate	35 ± 18[a] (5)
Quisqualate	12 ± 1.6 (4)
L-Glutamate + L-[^{35}S]cystine 10 μM	110 ± 19[a] (6)
L-Glutamate + L-[^{35}S]cystine 100 μM	450 ± 140[a] (5)
	K_a L-[^{3}H]Glutamate μM
L-Glutamate	30 ± 15 (3)
L-Cystine	23 ± 4 (3)
Quisqualate	5 ± 1[a] (3)

N18-RE-105 cells were incubated with radiolabeled cystine or Glu for 20 min in the presence or absence of unlabeled inhibitors (0.1–300 μM), and radiolabel uptake was determined as described in Experimental Procedures. K_t values of saturable L-[^{35}S]cystine (0.3–300 μM) or L-[^{3}H]glutamate (1–300 μM) transport and the apparent affinities (K_a) of unlabeled inhibitors (cystine, Glu, or Quis) for L-[^{35}S]cystine (5–15 nM) or L-[^{3}H]glutamate (100 nM) transporters are presented, as are the apparent affinities of Glu for 10 μM or 100 μM L-[^{35}S]cystine uptake. Values are the means ± standard deviation of (n) pooled separate duplicate experiments for K_a values and means ± SEM of (n) separate duplicate experiments for K_t values.
[a] $P < 0.05$; repeated measures analyses of variance were performed comparing inhibition of L-[^{35}S]cystine (5–15 nM) uptake by cystine with that of Glu or Quis; comparing inhibition of L-[^{35}S]cystine (5–15 nM) uptake by Glu with that of 10 μM L-[^{35}S]cystine + Glu or 100 μM L-[^{35}S]cystine + Glu; and comparing K_a values for inhibition of L-[^{3}H]glutamate (100 nM) uptake by Glu with that of Quis and cystine.

Inhibition of L-[^{35}S]Cystine Uptake in N18-RE-105 Cultures

Unlabeled cystine, Glu, or Quis competitively inhibited uptake of 5–15 nM L-[^{35}S]cystine (specific activity 354–866 Ci/mmol) with apparent affinities for the uptake site of 7.5 ± 1.6 μM (cystine), 35 ± 18 μM (Glu), and 12 ± 1.7 μM (Quis) (Table 2; Figure 3). Quis and Glu inhibition of L-[^{35}S]cystine uptake had average n_H values of 0.97 ± 0.1 and 0.95 ± 0.3. Curves for inhibition of L-[^{35}S]cystine uptake by cystine had n_H values averaging 0.78 ± 0.1. When 10 and 100 μM concentrations of un-

Table 3. Ionic Dependence of L-[^{35}S]Cystine Uptake

Ionic Manipulation	% Control Uptake: 10 μM L-[^{35}S]Cystine	1000 μM L-[^{35}S]Cystine
1.8 mM Cl^-	57 ± 7[a]	90 ± 9
7.2 mM Cl^-	64 ± 8[a]	94 ± 5
Low Na^+	77 ± 7	56 ± 6[a]
Low Ca^{2+}	92 ± 15	81 ± 5
Depolarization	127 ± 5[a]	67 ± 34

The ionic dependence of 10 μM and 1000 μM radiolabeled cystine uptake was evaluated using media containing 5–15 nM L-[^{35}S]cystine and supplemented with either 10 μM or 1000 μM cystine. Uptake was expressed as a percentage of control uptake (normal Ca^{2+}, Na^+, and Cl^-; see Experimental Procedures). Solutions were prepared as described in Experimental Procedures: low Ca^{2+} (<10 μM), low Na^+ approximately 15 mM, Cl^- concentrations as indicated. Depolarization was for 24 hr with 50 μM veratridine. Values are the means ± SEM of 3 separate experiments performed in triplicate.

[a] $P < 0.05$ (two-tailed paired t-test comparing uptake in control cultures with those in altered ionic composition).

labeled cystine were used (specific activity 0.5 mCi/mmol and 0.05 mCi/mmol, respectively), the apparent affinity for Glu was decreased to 110 ± 19 and 450 ± 140 μM ($P < 0.05$), respectively (Table 2), consistent with competitive inhibition. The n_H values for 10 μM L-[^{35}S]-cystine + Glu and 100 μM L-[^{35}S]cystine + Glu, were 0.97 ± 0.04 and 0.96 ± 0.05, respectively. None of the n_H values differed significantly from unity ($P > 0.1$).

Inhibition of L-[^{3}H]Glutamate Uptake in N18-RE-105 Cultures

Unlabeled Glu, Quis, and cystine competitively inhibited the uptake of 100 nM L-[^{3}H]glutamate (specific activity, 52 Ci/mmol) with an apparent affinity for unlabeled agonist of 30 ± 15 μM (Glu), 4.6 ± 1 μM (Quis), and 23 ± 4 μM (cystine) (Table 2; Figure 3). Glu, Quis, and cystine inhibition of L-[^{3}H]glutamate uptake had average n_H values of 0.81 ± 0.15, 0.85 ± 0.15, and 0.96 ± 0.07, respectively. None of the n_H values differed significantly from unity ($P > 0.1$).

Ionic Dependence of L-[^{35}S]Cystine Uptake in N18-RE-105 Cultures

The effects of Ca^{2+}, Cl^-, and Na^+ on the high affinity uptake of cystine were determined using ionic replacement of 10 μM L-[^{35}S]cystine (specific activity 0.5 Ci/mmol) uptake medium (Table 3). Removal of Ca^{2+} from incubation medium (normal Ca^{2+} = 1.8 mM) with the addition of EGTA did not have a significant effect on the uptake rate of 10 μM L-[^{35}S]cystine (Table 3), nor did it reduce the recovery of cellular protein. In contrast, reduction of extracellular Cl^- to 1.8 mM (normal Cl^- = 140 mM) significantly reduced uptake of 10 μM L-[^{35}S]cystine to 57% ± 7% of control (Table 3). In contrast, low Na^+ medium (approximately 15 mM Na^+; normal Na^+ = 140 mM) did not significantly affect uptake of 10 μM L-[^{35}S]cystine (Table 3).

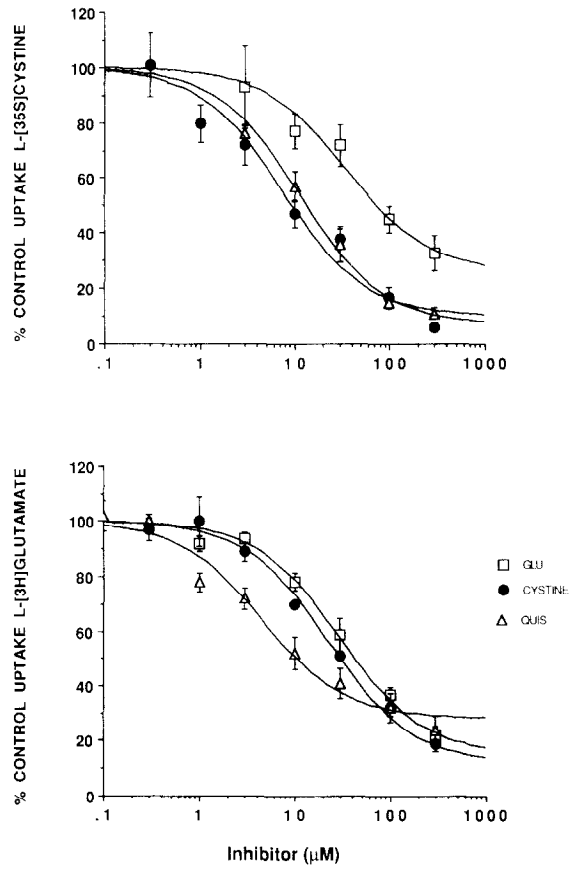

Figure 3. Inhibition of L-[^{35}S]Cystine and L-[^{3}H]Glutamate Uptake into N18-RE-105 Cells by Unlabeled Substrates

Experiments were performed as in Table 2 with the indicated concentration of unlabeled inhibitors added. Calculated K_i values from these data are presented in Table 2.

Specificity of L-[^{35}S]Cystine Uptake Inhibition by Acidic Amino Acids in N18-RE-105 Cells

Reported agonists and antagonists at Glu receptors (Watkins and Evans, 1981) and transport sites (Berry et al., 1988; Kessler et al., 1987; Zaczek et al., 1987a, 1987b) were tested at 1 mM concentrations for inhibition of L-[^{35}S]cystine uptake (15 nM; specific activity 354 Ci/mmol) (Table 4). Besides cystine itself, only compounds that were toxic to N18-RE-105 cells significantly reduced uptake: Quis, Glu, and ibotenate. In contrast, the nontoxic Glu analogs N-methyl-D-aspartate (NMDA), D-2-aminophosphonovalerate (D-APV), kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and DL-2-aminophosphonohexanoate (DL-APH) produced less than 10% inhibition, and DL-2-aminophosphonobutyrate <-APB), D-APB, and aspartate demonstrated modest inhibition that was not statistically significant.

Glutathione Levels in N18-RE-105 Cultures

Cellular levels of glutathione (oxidized and reduced, GSH/GSSG) decreased in a time-dependent fashion during Glu exposure (Figure 4). After only 4 hr of 10 mM Glu exposure (which is prior to the commencement of cell lysis), GSH/GSSG levels were reduced by 50%. After 8

Table 4. Effect of Glu Agonists and Antagonists on the Uptake of 15 nM L-[^{35}S]Cystine

Substrate	% Control Uptake
Quisqualate	17 ± 4[a] (8)
Glutamate	24 ± 8[a] (9)
Ibotenate	33 ± 10[a] (3)
DL-APB	64 ± 12 (4)
Aspartate	77 ± 8 (5)
D-APB	83 ± 14 (3)
AMPA	94 ± (2)
DL-APH	95 ± 10 (3)
D-APV	97 ± 10 (3)
Kainate	107 ± 22 (3)
NMDA	109 ± 17 (3)

N18-RE-105 cells were incubated with 15 nM L-[^{35}S]cystine or 15 nM L-[^{35}S]cystine + 1 mM of the unlabeled inhibitors for 20 min, and radiolabel uptake was determined as described in Experimental Procedures. Control uptake is the uptake of 15 nM L-[^{35}S]cystine in the absence of any inhibitor. Values are the mean ± SEM of (n) separate duplicate experiments.
[a] $P < 0.05$ (two-tailed paired t-test).

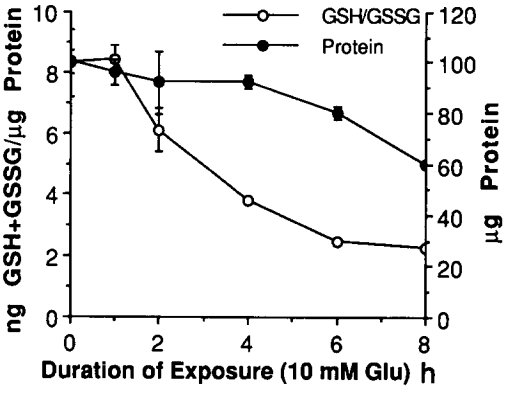

Figure 4. Time Course of Glutathione Depletion by Glutamate
N18-RE-105 cells were exposed to 10 mM Glu for the duration indicated (in hours). The levels of cellular acid-soluble GSH + GSSG and acid-precipitable cellular protein were determined as described in Experimental Procedures. The results are means ± SEM from a representative time course performed in triplicate.

hr of exposure, when cell lysis had begun, GSH/GSSG levels had fallen to 32% of control. GSH/GSSG levels were also measured in cultures pretreated with agents that have previously been shown to protect against the cytotoxic effects of Glu in this cell line (Murphy et al., 1988a, 1988b; Murphy et al., 1989). No significant change in GSH/GSSG levels was noted in the presence of vitamin E and the lipooxygenase inhibitor nordihydroguaiaretic acid, in low Ca^{2+}, or after depolarization with veratridine, when compared with matched control cultures or matched Glu-treated cultures (Table 5).

Intracellular Peroxides in N18RE-105 Cultures

An indication of intracellular peroxide formation upon Glu treatment was obtained by labeling the cells with the probe 2,7-dichlorofluorescin diacetate, which becomes trapped in cells and fluoresces upon oxidation. Bright-field and fluorescent microscopy (Figure 5) of >200 cells from each of 3 separate experiments revealed a significantly greater percentage of fluorescent cells in Glu-treated cultures (66% ± 10% after 10 mM Glu for 5–7 hr) than in matched control cultures (14% ± 7%; $P < 0.01$).

Sensitivity of Mature Primary Hippocampal Neurons to Low Cystine Toxicity

To assess whether a similar mechanism of cytotoxicity may affect primary neurons, hippocampal neurons in culture were studied. After 12 hr of low cystine treatment, some neurons appeared swollen but most were histologically intact (Figure 6). However, after 18 hr of low cystine exposure, neurons displayed numerous surface blebs and necrotic processes, and by 24–30 hr, few intact cells remained (1–3 µM cystine). The underlying confluent glial mat was largely unaffected by the low cystine medium. This was evidenced by two criteria, absence of trypan blue staining of flat cells and retention of cellular LDH content. About 10% of the total LDH in

Table 5. Cellular Glutathione Levels

Treatment	GSH + GSSG ng per µg of Protein		
	No Added Glu	10 mM Glu, 8 hr	Cytotoxicity % of Control
Control	7.5 ± 1.2	2.4 ± 0.8[a]	100
Low Ca^{2+}	6.4 ± 1.1	2.2 ± 0.7[a]	−3 ± 3[a]
Depolarization	7.6 ± 1.8	1.6 ± 0.7[a]	30 ± 11[a]
NDGA	10.6 ± 2.0	2.2 ± 0.8[a]	3 ± 1[a]
Vitamin E	10.7 ± 2.4	2.2 ± 0.9[a]	0 ± 6[a]

Cellular GSH + GSSG levels were determined enzymatically for N18-RE-105 cells treated with 10 mM Glu for 8 hr and/or conditions associated with reduction in Glu toxicity. Values are expressed as ng of GSH per µg of cellular protein and are the means ± SEM of at least 3 experiments performed in duplicate. All treatments were initiated 12 hr before and were continued during Glu exposure. Low Ca^{2+} medium was prepared without added Ca^{2+} and contained 0.5 mM EGTA; depolarization was with 50 µM veratridine; nordihydroguaiaretic acid (NDGA) was added at 5 µM; and vitamin E was added at 100 µM. Cytotoxicity measurements were performed on different cultures after 24 hr of 10 mM Glu exposure. Values are the means ± SEM of at least 3 separate duplicate experiments; Glu cytotoxicity ranged from 50%–80%. None of the treatments significantly altered GSH levels compared with matched control cultures (with and without added Glu; $P > 0.1$). However, the difference between untreated and Glu-treated cultures was significant for each treatment.
[a] $P < 0.05$ (two-tailed unpaired t-test, performed for GSH + GSSG and cytotoxicity measurements).

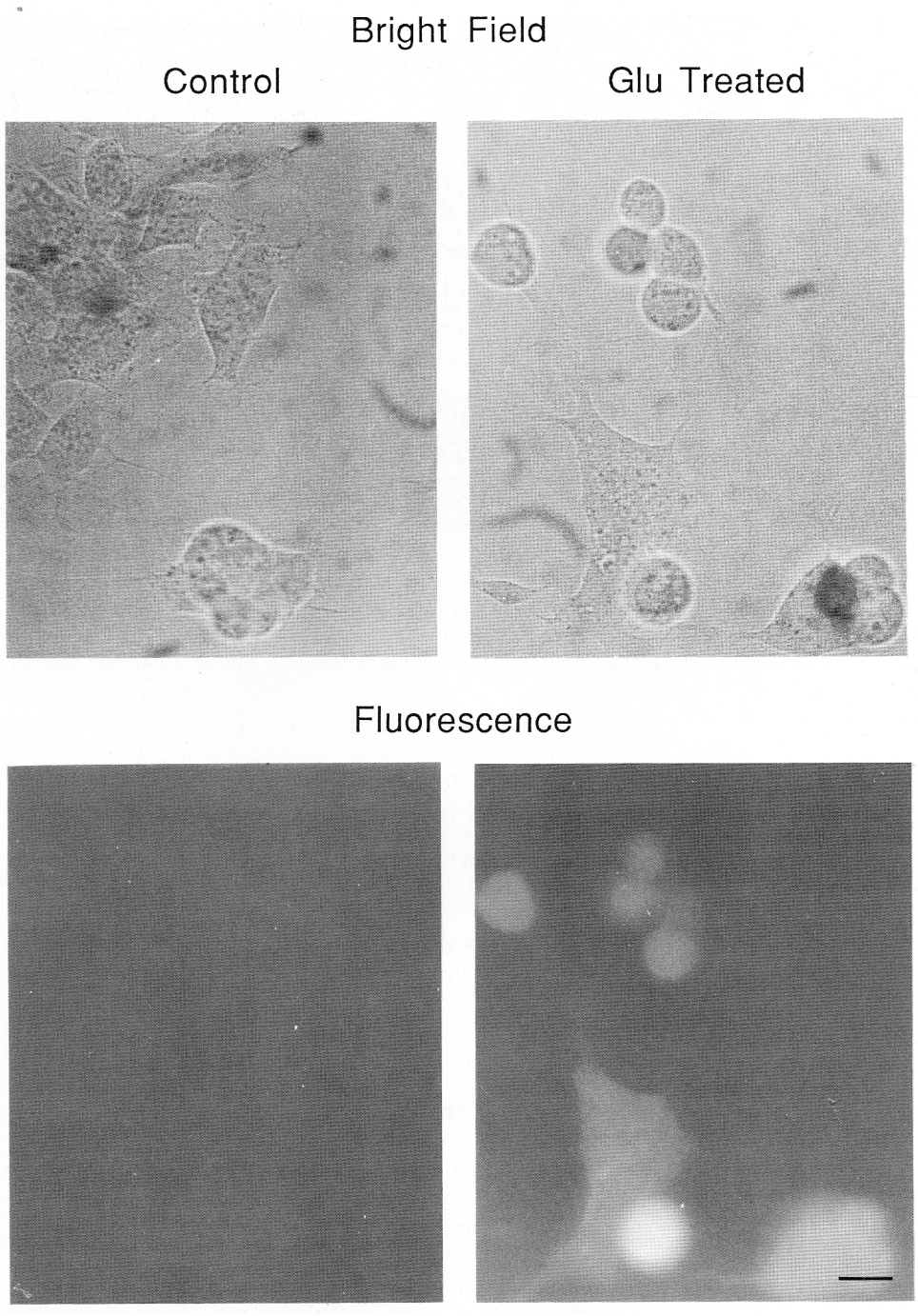

Figure 5. Glutamate-Stimulated Intracellular Peroxide Formation

Formation of peroxides after 8 hr of 10 mM Glu exposure was detected using 2,7-dichlorofluorescin diacetate oxidation and fluorescence in N18-RE-105 cells. Representative fluorescence and light photomicrographs of Glu-treated and control cultures are presented. Bar, 15 μm.

the culture wells was neuronal (rather than glial), as estimated by the amount released after exposure to NMDA agonists that selectively kill neurons (Choi et al., 1987). After low cystine exposure (1 μM for 24 hr), greater than 90% of the neurons in the cultures had died (estimated by counting phase-bright cells with obvious processes; see Experimental Procedures), while the amount of LDH released was less than 10% of the total LDH in the culture. The cystine concentration at which 50% of the neurons were viable (24 hr exposure) was approximately 3 μM, using either cell counting or LDH release as the method of quantitation (Table 6). Some glial toxicity was observed at later times (72 hr) of exposure to cystine-free medium.

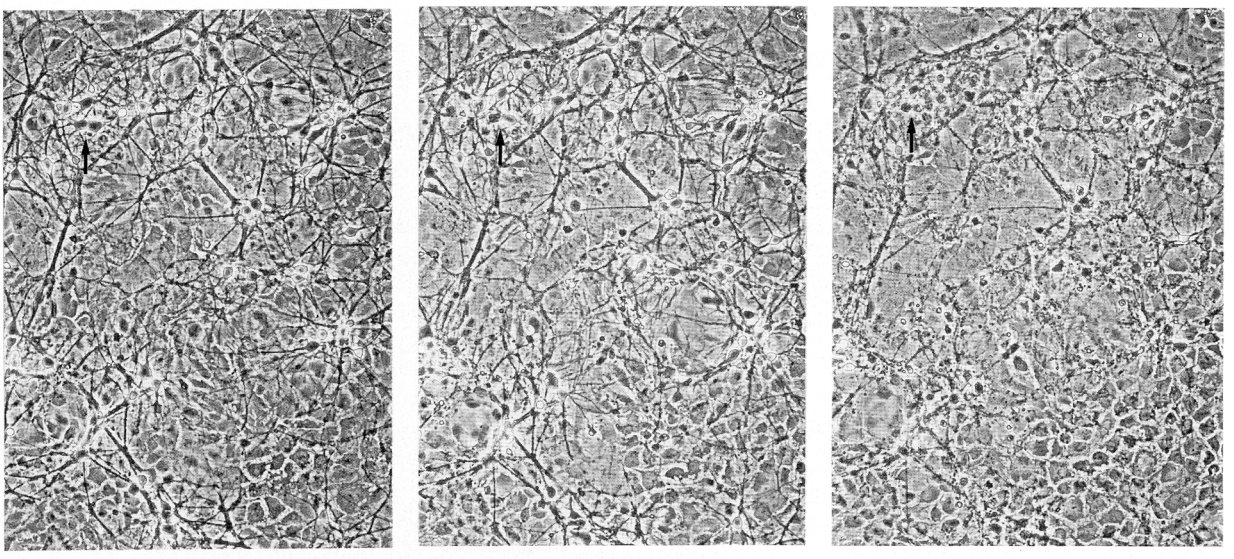

Figure 6. Medium Depleted in Cystine (1 μM) Produces Delayed Toxicity of Hippocampal Neurons
Shown is a field of hippocampal neurons and glia after exposure to cystine-depleted medium for the indicated periods of time.

Discussion

N18-RE-105 cells cultured in medium deficient in cystine underwent delayed degeneration (Figure 1). The cytopathological alterations, time course, and Ca^{2+} dependence of cell death were similar to those observed with Glu-induced cytotoxicity in medium with a normal concentration of cystine (Murphy et al., 1988a). The cytotoxic potencies of Glu and Quis were dependent on the concentration of cystine in the culture medium (Figure 1). Thus, when the cystine concentration was lowered, the cytotoxic potencies of Glu and Quis were increased, whereas an increase in medium cystine decreased their cytotoxic potency (Figure 1). Furthermore, Glu and Quis competitively inhibited the uptake of L-[^{35}S]cystine into N18-RE-105 cells. The alterations in the cytotoxic potency of Glu caused by changing the cystine concentration in the medium can be predicted by using the K_a values determined from uptake studies (Figure 7). The competitive interaction between Glu and cystine accounts for the observation that millimolar concentrations of Glu were required to produce cytotoxicity in standard culture medium (DMEM) containing 200 μM cystine.

Table 6. Concentration Dependence of Low Cystine Toxicity of Primary Hippocampal Neurons

Cystine (μM)	% Cell Death	
	LDH Release	Counting Cells
100	0	12 ± 3
30	−8 ± 8	15 ± 2
10	5 ± 11	19 ± 4
3	47 ± 10[a]	63 ± 8[a]
1	100[a]	92 ± 2[a]

Neuronal toxicity was determined by LDH release and by counting neurons (with cell death expressed as the percentage of necrotic cells versus total viable and necrotic cells) after 24–30 hr of exposure to medium containing the indicated concentration of cystine. Results are the means ± SEM of 3 separate experiments performed in duplicate.
[a] $P < 0.05$ (two-tailed paired t-test comparing cell death at various concentrations of cystine with that at 100 μM).

The relationship between cytotoxicity and cystine concentration could be fit to the equation for a rectangular hyperbola and had a Hill coefficient of 1.06, suggesting that the primary event in cytotoxicity was reduction in occupancy of the carrier by cystine. If this inference is valid, the relationship between occupancy at the cystine carrier and cytotoxicity should be linear with a slope of −1. In 4 separate experiments in which the cystine concentration in the medium was varied (5 to 1000 μM), the slope was −1.1 (R^2 = 0.99) for percent cell death versus percent occupancy (Figure 7). For 3 experiments, varying the concentration of cystine (20 to 1000 μM) in the presence of 1 mM Glu resulted in a slope of −1.27 (R^2 = 0.98) for percent cell death versus percent occupancy. While a slight difference in slope and X-intercept was observed, this was not likely to be significant, since the calculated occupancy depends on several experimentally determined parameters that have some associated error. The similarity between the K_a for cystine transport, 7.5 μM, and the K for cystine toxicity, 11 μM, supports the conclusion that the two phenomena are closely related.

Primary cultures of hippocampal neurons are also vulnerable to reduced cystine in their medium, with the same concentration dependence, delayed degeneration, and cytopathology observed in the N18-RE-105 cell line. Glial cells were unaffected during the time frame

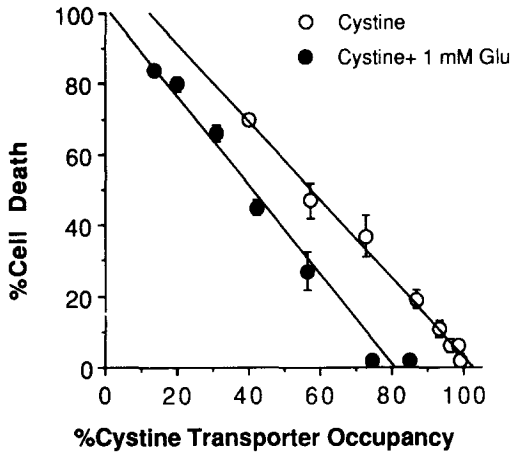

Figure 7. The Relationship Between Occupancy of the Cystine Uptake Site by Cystine and Toxicity of N18-RE-105 Cells

Shown are two methods for reducing transporter occupancy by cystine: varying cystine concentration or varying cystine concentration in the presence of 1 mM Glu. Occupancy was calculated based on parameters derived from L-[^{35}S]cystine uptake inhibition experiments:

$$\% \text{ occupancy} = 100 \cdot L_c/(L_c + K_c) \text{ for cystine, and}$$

$$\% \text{ occupancy} = R_c \cdot L_c/(L_c + K_c) + R_g \cdot L_c/(L_c + K_c \cdot (1 + L_g/K_g)) \text{ for cystine + Glu}$$

K_c is the K_a of cystine, and K_g is the K_a of Glu, R_c is the percentage of cystine uptake sites that are not inhibited by Glu (~25%), R_g is the percentage of cystine uptake sites that are Glu-sensitive (~75%), L_c is the concentration of cystine, and L_g is the concentration of Glu. Means ± SEM of 3 separate triplicate experiments with 5–500 μM cystine or 20–1000 μM cystine + 1 mM Glu (points with error bars) or means of 2 separate triplicate experiments (points without error bars).

that resulted in profound neuronal degeneration. Studies in progress are addressing the role of Glu-induced inhibition of cystine transport on cytotoxicity in primary neuronal cultures.

Cystine and Glu both have a net charge of −1 at pH 7.4 (Bannai, 1984) and can be transported by high affinity (micromolar affinities) antiporters, which exchange molecules of like charge in a manner independent of metabolic energy. Extracellular cysteine is rapidly oxidized to cystine, then taken up, reduced, and used in the synthesis of GSH or protein (Bannai, 1984). GSH plays a major role in cellular metabolism as an antioxidant and free radical scavenger, and GSH depletion results in cell degeneration due to oxidative stress (Halliwell, 1987). For example, Bannai et al. (1977), using fibroblasts, found that reducing cystine levels in the culture medium caused delayed cell degeneration. This cytotoxicity was prevented by free radical scavengers, suggesting that oxidative events were occurring. Bannai also characterized the uptake of cystine into fibroblasts and found it to be inhibited by Glu. Additionally, the uptake of Glu was inhibited by cystine, suggesting that they shared a common carrier site. However, the affinities of cystine and Glu at this site were at least one-fifth that reported for N18-RE-105 cells (Bannai and Kitamura, 1980). May and Gray (1985) have shown in primary fibroblast cultures that addition of Glu to the medium caused a reduction in cystine uptake and a decrease in cellular GSH concomitant with cell death. However, the toxicity of Glu if fibroblasts was not apparent until occupancy of the cystine antiporter was reduced to less than 5% (fibroblast transport kinetic constants from Bannai and Kitamura, 1980), indicating that nonneuronal cells are far less sensitive to this type of cytotoxicity.

The apparent affinities of Glu, cystine, and Quis for their uptake site, determined with L-[^{35}S]cystine, were in good agreement with those determined using L-[^{3}H]glutamate, suggesting that Glu, Quis, and cystine bind to the same carrier. High affinity sites were of primary interest since their affinities correlated best with the observed cytotoxic effect of low cystine and added Glu. However, we do not exclude the possibility of lower affinity transport sites for Glu and for cystine. In support of the existence of more selective, lower affinity sites mediating Glu and cystine transport, the uptake of L-[^{35}S]cystine could not be completely blocked by Glu, and L-[^{3}H]glutamate uptake was not completely blocked by cystine.

The uptake system for cystine in N18-RE-105 cells is similar to that for the Na^+-independent and Cl^--dependent transport of Glu described in neurons (Table 4) (Berry et al., 1988; Kessler et al., 1987; Zaczek et al., 1987a, 1987b) but has a 5-fold higher affinity than the cystine/Glu antiporter in fibroblasts (Bannai and Kitamura, 1980). Only substrates that significantly reduced L-[^{35}S]cystine uptake (e.g., Quis, Glu, and ibotenate) were appreciably cytotoxic (Murphy et al., 1988a). Similarly, Quis, ibotenate, and Glu are among the most potent compounds at inhibiting Cl^--dependent L-[^{3}H]glutamate binding/sequestration in membrane preparations from both rat brain and N18-RE-105 cells (Berry et al., 1988; Kessler et al., 1987; Zaczek et al., 1987a, 1987b; Malouf et al., 1984). Notably, the N18-RE-105 cell line lacks Na^+-dependent high affinity uptake of Glu, which is expressed in subpopulations of brain neurons and glia (Malouf et al., 1984).

Many different mechanisms of cell death may share a common final oxidative event; examples are menadione (Di Monte et al., 1984), Ca^{2+} ionophores (Schanne et al., 1979), and starvation (Saez et al., 1987). Our findings indicate that free radicals were produced 5–7 hr after Glu treatment of N18-RE-105 cells and that several free radical scavengers can protect against the cytotoxic effect of high Glu concentrations. Both the depletion of cellular GSH and the formation of free radical appeared to precede cell death. However, GSH depletion by Glu is not sufficient in itself to cause cell lysis, since each of the protective treatments—low Ca^{2+}, lipooxygenase inhibitor, depolarization, and vitamin E—failed to protect against the Glu-induced depletion in GSH levels, although they protected against cytotoxicity.

With decreased levels of GSH, the ability of N18-RE-105 cells to scavenge free radicals formed by Ca^{2+}-dependent pathways, i.e., lipooxygenases and phospholipases (Samuelsson et al., 1987; Baba et al., 1986),

may be impaired. Low Ca^{2+} medium would prevent the activation of these enzymes. Consistent with this interpretation, the lipooxygenase inhibitors nordihydroguaiaretic acid and phenidone and the phospholipase A_2 inhibitors quinacrine and 4-bromo phenacyl Br^- reduce the cytotoxicity of Glu in N18-RE-105 cells (Murphy et al., 1989). Alternatively, altered sulfhydryl redox status has been reported to change the function of proteins involved with Ca^{2+} disposition; for example, the reduced activity of Ca^{2+} ATPase in hepatocytes is caused by oxidative stress (Di Monte et al., 1984). The observed protective effects of divalent metals such as Cd^{2+} against low cystine-type cell death and glutamate-induced cell death (Murphy et al., 1988b) suggest that intracellular oxidants may be able to produce prolonged activation of voltage-sensitive Ca^{2+} channels by damaging their gating mechanism. Alternatively, Cd^{2+} could interfere with other Ca^{2+}-dependent processes. Thus, the protective effects of these treatments suggest that GSH depletion renders the cells vulnerable to secondary events, which can be blocked at several different control points, that lead to cell lysis.

Plasma cystine and Glu concentrations are from 80 to 140 μM (Battistin et al., 1971; Perry et al., 1975); thus uptake via low affinity, high capacity systems may dominate in the periphery. In contrast, the cerebrospinal fluid (CSF) concentration of cystine in mammals is between 0.1 and 0.3 μM (Perry et al., 1978; Gjessing et al., 1972). While the concentration of cystine in CSF is below that required for survival of neurons in primary culture, the levels in the perineural space may be higher. Nevertheless, the CSF values suggest that the extracellular concentrations of cystine are substantially lower in the brain than in the periphery. In mouse brain, tissue concentrations of free Glu are approximately 10 mM, tissue cystine is approximately 30 μM (Battistin et al., 1971), and brain extracellular Glu concentrations are between 1.5 and 15 μM (Benveniste et al., 1984; Perry et al., 1978; Gjessing et al., 1972). Since antiporters move substrates down their concentration gradients, it is likely that the neuronal cystine/Glu antiporter functions to transport Glu out and cystine in.

During experimentally induced brain ischemia, extracellular Glu concentrations have been shown to increase to levels 800% of control (Benveniste et al., 1984), which would inhibit cystine uptake. Consistent with this inference, ischemia reduces brain GSH levels (Rehncrona et al., 1980). The neuronal toxicity of kainate is indirect and requires the integrity of glutamatergic afferents (McGeer et al., 1978; Biziere and Coyle, 1979). Kainate releases Glu presynaptically (Lehmann et al., 1983; Ferkany and Coyle, 1983), which would inhibit the neuronal uptake of cystine, possibly explaining the indirect toxicity of kainate. Furthermore, free radical scavengers protect against kainate-induced neurotoxicity in primary cell cultures (Dykens et al., 1987). While NMDA does not directly affect the cystine carrier (Table 4), activation of its receptors causes influx of Ca^{2+} via Mg^{2+}-sensitive channels (Mayer et al., 1984; Jahr and Stevens, 1987). Elevated intracellular Ca^{2+} produces oxidative stress (Schanne et al., 1979), which may make neurons more dependent on cystine uptake for GSH synthesis, possibly unmasking a *contribution* of Glu-sensitive cystine transport to the delayed toxicity of NMDA agonists in vivo. Thus, we speculate that inhibition of cystine uptake by elevated concentrations of extracellular Glu during various types of brain insults, such as ischemia (Simon et al., 1984), might reduce the ability of neurons to maintain redox homeostasis and ultimately *contribute* to cell death. Whether a role for cystine uptake inhibition exists in neurodegenerative disease awaits further studies in vivo and in primary cultures.

Experimental Procedures

Materials

Tissue culture medium (DMEM; #430-2100) and fetal calf serum were purchased from GIBCO; L-[^{35}S]cystine (354–866 Ci/mmol) and L-[^{3}H]glutamate (51.9 Ci/mmol) from New England Nuclear; 35 mm tissue culture dishes from Corning; 24 well tissue culture plates from Costar; Quis, D-APV, and D-APB, from Cambridge Research Biochemicals; ibotenate from Regis Biochemicals; NMDA, cystine, kainate, Na^+ gluconate, K^+ gluconate, N-methyl-D-glucamine, bovine serum albumin (fraction V), veratridine, nordihydroguaiaretic acid, α-tocopherol (vitamin E), and EGTA from Sigma; DL-APH and AMPA from Research Biochemicals Incorporated; Triton X-100 and DL-APB from Calbiochem; bicinchoninic acid reagent from Pierce; DTNB, HEPES, GSSG reductase (600 U/ml), and GSH from Boehringer Mannheim; and 2,7-dichlorofluorescin diacetate from Molecular Probes. All other chemicals were purchased from standard sources.

N18-RE-105 Cell Culture and Cytotoxicity Quantitation

N18-RE-105 cells were grown as described previously in DMEM containing 5% fetal calf serum and hypoxanthine, aminopterin, and thymidine (control culture medium, 200 μM cystine) (Malouf et al., 1984). For cytotoxicity studies, cells were subcultured from confluent 75 cm^2 flasks and plated on 35 mm culture dishes at a density of 20,000 cells per dish in 1 ml of the above medium (unless otherwise noted). After culturing for 24 hr, the medium was removed and replaced with medium (formulation described above) containing the indicated amounts of cystine and/or Glu agonists.

After the times indicated in the presence of medium with altered cystine concentration and/or Glu agonists (0–24 hr), cytotoxicity was monitored by phase-contrast microscopy and quantitated by the release of the cytosolic enzyme LDH into the culture medium. LDH is an easily measured, soluble enzyme, the activity of which is proportional to cell number (Schnaar et al., 1978). Experiments with N18-RE-105 cells demonstrate that LDH activity released into the culture medium is stable for at least 72 hr (data not shown). Complete analysis of cell lysis required measurement of the LDH activity in three pools: that released into the medium, that in any nonadherent intact cells (usually a small pool), and that in substratum-attached intact cells. After exposure to toxic conditions, the medium was collected from each culture plate and centrifuged at 200 × g for 5 min (to sediment any nonadherent cells). The supernatant was transferred to a fresh tube, and any pelleted cells were lysed in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100 (lysis buffer). Cells remaining on the plate were lysed in 1 ml of lysis buffer. LDH activity in the culture medium (supernatant) and in the cell lysates was measured spectrophotometrically (Schnaar et al., 1978). The percent cell death was defined as the amount of LDH released into the culture medium divided by the total LDH activity in the culture (medium + lysed detached cells + lysed attached cells).

Cystine-free DMEM was prepared using a published formulation (Eagle, 1955; Smith et al., 1960) except that cystine was eliminated. Cystine was added to media by dilution of a 100 mM stock dissolved in deionized water by addition of NaOH. After the addition of the

cystine stock to media, the pH was adjusted as necessary by the addition of HCl.

Primary Cell Culture and Toxicity

Cultures of mixed primary hippocampal neurons and glia were prepared by the method of Huettner and Baughmann (1986). Briefly hippocampi were dissected from 17 day gestation rat fetuses and dissociated enzymatically with papain. The cells were then plated on 24 well plates at a density of 6×10^5 cells per ml in 1.2 ml of minimal essential medium supplemented with penicillin, streptomycin, 10% fetal bovine serum, and 5% horse serum. The cells were cultured for 7 days, at which time they were treated with 5-fluoro-2-deoxyuridine (10 μM) and uridine (100 μM). The cells were then cultured for 7 more days (with feeding every 3 days by half replacement of medium, with medium containing horse serum, but lacking fetal serum) and were used for experiments between 14 and 21 days in vitro. Cultures were washed twice in DMEM without cystine (200 μM cystine medium is normally present). After the final wash, serum-free DMEM containing various concentrations of cystine (1–100 μM) was added to the cultures, which were then incubated for 24–30 hr at 37°C. In some cultures, fields were selected at random and their locations were marked and photographed over the exposure period (between photographs cells were returned to the incubator). After the exposure period, the medium was removed and the cells were lysed for LDH measurement as previously described for N18-RE-105 cells.

Since the primary hippocampal cultures contained both neurons and glia, neuronal toxicity was quantitated as follows. Exposure of cultures (24–30 hr) to medium containing 1 μM cystine resulted in LDH release which was similar to that produced by a 5 min or continuous exposure to NMDA or Glu (1 mM) and was defined as maximal cell death. The amount of LDH released into normal cystine-containing medium for the same time was considered basal release (Koh and Choi, 1988). Toxicity was expressed as a percentage of maximum (neuronal) LDH release over basal.

Toxicity was also determined by counting viable and nonviable neurons and defining percent cell death as the percentage of nonviable neurons. Neurons were identified as large, phase-bright cells with obvious processes. Identification was confirmed in some cultures using antisera to neuron-specific enolase, and a statistically significant reduction in cells positive for neuron-specific enolase was observed following low cystine exposure (data not shown). Neurons were considered dead if they were not phase-bright, had ruptured cell membranes, and displayed severed processes. Viability was routinely checked by trypan blue exclusion. However, counting viable and nonviable neurons may have underestimated cell death at the lowest cystine concentration used (1 μM), since some necrotic cells had completely disintegrated and thus were unable to be counted (see Figure 6).

L-[^{35}S]Cystine and L-[^{3}H]Glutamate Uptake

Amino acid uptake was evaluated using cells grown as described above. Briefly, N18-RE-105 cells were plated at a density of 4000 cells per well in 0.2 ml of medium into 24 well plates. Cells were grown for 24–48 hr prior to measurement of uptake. Uptake was measured using a modification of the method of Gazzola et al. (1981). Medium was removed from each well by aspiration, and the adherent cells were washed once with 2 ml of a modified Hank's balanced salt solution (HBSS) containing 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.4 mM KH_2PO_4, 0.6 mM $Na_2HPO_4(7H_2O)$, 10 mM Na+HEPES, 4.1 mM $NaHCO_3$, and 5 mM glucose (pH 7.4 and 340 mosm). The washed cells were preincubated for 15–20 min in 500 μl of HBSS per well at 37°C, then washed again as described above. The medium was removed as described above and replaced with 300 μl of HBSS containing 5–15 nM L-[^{35}S]cystine (354–866 Ci/mmol) or 100 nM L-[^{3}H]glutamate (51.9 Ci/mmol) and the indicated amounts of unlabeled amino acids. After incubation at 37°C for the indicated times, the medium was removed and the cells were washed rapidly three times with 2 ml of ice cold HBSS, without added amino acids. The cells were then lysed with 200 μl of lysis buffer, and 100 μl of lystate was removed for radioactivity determination by liquid scintillation spectroscopy counting in an aqueous accepting fluor. The remaining 100 μl was retained for subsequent protein determination by the bicinchoninic acid reagent method (Smith et al., 1985) with bovine serum albumin as standard. Ca^{2+}-free uptake buffer was made by omitting Ca^{2+} from HBSS and adding 0.5 mM Na^+EGTA (pH 7.4). Low Cl^- buffer (1.8 and 7.2 mM) was prepared by replacing added KCl and NaCl in HBSS with the appropriate gluconate salts. Low Na^+ buffer (approximately 15 mM added Na^+) was made by replacing NaCl in HBSS with N-methyl-D-glucamine Cl^-.

Glutathione Levels

Cellular GSH + GSSG levels (total amount of both forms) were measured using a modification of the method of Tietze (1969). N18-RE-105 cells were plated on 60 mm culture dishes and grown as described above, washed once with HBSS, collected with a rubber policeman in 1 ml of 3% $HClO_4$, removed to a microcentrifuge tube, and placed on ice for 15 min. The tubes were centrifuged (10 min at 7400 × g at 4°C) to remove precipitated protein, and the supernatants were transferred to fresh test tubes containing 0.5 M KOH and 9 mM $Na_2B_4O_7$ (0.8–0.9 ml, adjusted to pH 7.5). The tubes were placed on ice for 10 min to allow precipitation and settling of $KClO_4$. An aliquot (0.25 ml) of the neutralized supernatant was added to 0.25 ml of 0.1 M potassium phosphate and 5 mM EGTA (pH 7.5) in a 1 ml plastic cuvette, then 0.4 ml of a solution containing 0.1 mg/ml DTNB, 0.32 mg/ml NADPH, and 0.1 M phosphate buffer (pH 7.5) was added. GSH + GSSG was determined spectrophotometrically by measuring the change in A_{412} upon addition of 100 μl of GSSG reductase (8 U/ml). Experimental rates were compared with those determined using GSH standards treated in the same manner. Precipitated protein pellets (above) were air-dried and resuspended in 0.1 M NaOH for protein determination using the bicinchoninic acid reagent method (Smith et al., 1985).

Intracellular Peroxide Formation

Formation of intracellular peroxides was detected using 2,7-dichlorofluorescin diacetate, which is a nonfluorescent cell-permeable compound (Bass et al., 1983). Upon entering cells, the compound is de-esterified and is subject to oxidation by cellular peroxides to the fluorescent compound 2,7-dichlorofluorescein. Glu-treated (10 mM Glu, 5–7 hr) and control cultures were allowed to take up 10 μM 2,7-dichlorofluorescin diacetate (added as 10 mM stock in DMSO) for 10 min at 37°C. The cultures were then washed twice with HBSS, and cellular fluorescence was viewed in HBSS at 25°C using a Leitz fluorescence microscope with fluorescein filters. Cultures were compared, with treatment condition blind to the investigator, and fluorescence was determined qualitatively by counting cells first under bright-field and then under fluorescence and expressing results as the percent fluorescent cells.

Data Analysis

To determine K_t values, uptake data were fit to the following equations:

$$V = V_{max} \cdot S/(S + K_t) \quad \text{for cystine uptake}$$
$$V = V_{max} \cdot S/(S + K_t) + M \cdot S + B \quad \text{for Glu uptake}$$

in which V is the velocity of uptake in pmol/mg of protein per 20 min, V_{max} is the maximal saturable transport velocity, S is the concentration of radiolabeled substrate, M is the slope of a linear nonsaturable component, and B is the intercept of the linear component, using V_{max} and K_t as free parameters for cystine and V_{max}, K_t, M, and B as free parameters for Glu. Addition of a small nonsaturable linear component (13% of total uptake when [S] = K_t) to the equation for Glu uptake resulted in a significant improvement in the modeling for uptake data by the calculated extra sums of squares test (Munson and Rodbard, 1980) (data set 0–3000 μM Glu) compared with a single site, whereas addition of a second saturable uptake site to the model did not.

Apparent affinities (K_a) of unlabeled Glu, cystine, or Quis for the cystine and Glu carrier were calculated by measuring the uptake of 5–15 nM L-[^{35}S]cystine or 100 nM L-[^{3}H]glutamate in the presence of various concentrations of unlabeled inhibitor and estimating affinities with R and K_a as free parameters using the following equation:

$$\% \text{ control uptake} = 100 - ((R \cdot L)/(L + K_a))$$

in which R is the percentage of carriers that are sensitive to inhibitor, control uptake is that in the absence of any inhibitor, L is the unlabeled inhibitor concentration, and K_a is the apparent inhibitor affinity. Hill coefficients (n_H) were calculated using

$$\% \text{ control uptake} = 100 - ((R \cdot L^{n}{}_{H})/(L^{n}{}_{H} + K_a))$$

Hill coefficients were fitted with n_H and R as free parameters and with K_a values held constant (at the value retained by setting n_H = 1, which resulted in a significantly better fit by the criterion of the extra sum of squares test (Munson and Rodbard, 1980) than when the three parameters were allowed to vary). For calculation of inhibitor affinities and n_H coefficients, data from 3–6 experiments were pooled and analyzed by nonlinear regression and the results were presented as the mean ± standard deviation. All other data are presented as the mean ± SEM, unless noted otherwise. Affinities and n_H coefficients were also calculated for each individual experiment, and two-tailed unpaired t-tests were performed comparing the values for different inhibitors and/or radiolabeled substrates. Radiolabeled substrate concentrations were less than 0.5% of the measured K_t values for Glu or cystine. When the concentration of radiolabel is much less than the K_t, K_t and K_a are mathematically equivalent; thus apparent differences between values for K_t and K_a for a particular substrate are not of biological significance. Constants for cell death and uptake data were estimated by the least squares method, using an iterative curve-fitting computer program.

Acknowledgments

We would like to thank Patti Hill for excellent technical assistance with tissue culture, Dr. Mack Mitchell for helping us with the GSH/GSSG assay, Dr. Marilyn Sanders for providing the hippocampal cultures, Dr. Paul Manis for assistance with fluorescence methods, and Dr. Peter Maloney for helpful discussions on amino acid transport. T. H. M is supported by NIH predoctoral training grant GM0726, R. L. S. is supported by an American Cancer Society Research Award (FRA-280) and NIH grant HD14010, and J. T. C. is supported in part by United States Public Health Service Grant NS13584 and by a grant from the McKnight Foundation.

Received February 10, 1989; revised April 6, 1989.

References

Baba, A., Onoe, H., Ohta, A., and Iwata, H. (1986). Assay of phospholipase A2 activity of synaptic membranes using a phospholipid transfer protein: stimulation by depolarization. Biochim. Biophys. Acta *878*, 25–31.

Bannai, S. (1984). Transport of cystine and cysteine in mammalian cells. Biochim. Biophys. Acta *779*, 289–302.

Bannai, S. (1986). Exchange of cystine and glutamate across plasma membrane of human fibroblasts. J. Biol. Chem. *261*, 2256–2263.

Bannai, S., and Ishii, T. (1988). A novel function of glutamine in cell culture: utilization of glutamine for the uptake of cystine in human fibroblasts. J. Cell. Physiol. *137*, 360–366.

Bannai, S., and Kitamura, E. (1980). Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. J. Biol. Chem. *255*, 2372–2376.

Bannai, S., and Kitamura, E. (1981). Role of proton dissociation in the transport of cystine and glutamate in human diploid fibroblasts in culture. J. Biol. Chem. *256*, 5770–5772.

Bannai, S., Tsukeda, H., and Okumura, H. (1977). Effect of antioxidants on cultured human diploid fibroblasts exposed to cystine-free medium. Biochem. Biophys. Res. Commun. *74*, 1582–1588.

Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M. (1983). Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J. Immunol. *130*, 1910–1917.

Battistin, L., Grynbaum, A., and Lajtha, A. (1971). The uptake of various amino acids by the mouse brain in vivo. Brain Res. *29*, 85–99.

Benveniste, H., Drejer, J., Schousboe, A., and Diemer, N. H. (1984). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J. Neurochem. *43*, 1369–1374.

Berry, B., Boland, L. M., Hock, D. B., and Dingledine, R. (1988). L-Glutamate binding site on N18-RE-105 neuroblastoma hybrid cells is not coupled to an ion channel. J. Neurochem. *51*, 1176–1183.

Biziere, K., and Coyle, J. T. (1979). Effects of cortical ablation on the neurotoxicity and receptor binding of kainic acid in striatum. J. Neurosci. Res. *4*, 383–398.

Choi, D. W., Maulucci-Gedde, M. A., and Kriegstein, A. R. (1987). Glutamate neurotoxicity in cortical cell culture. J. Neurosci. *7*, 357–368.

Di Monte, D., Bellomo, G., Thor, H., Nicotera, P., and Orrenius, S. (1984). Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. Arch. Biochem. Biophys. *235*, 343–350.

Dykens, J. A., Stern, A., and Trekner, E. (1987). Mechanism of kainate toxicity to cerebellar neurons in vitro is analogous to reperfusion injury. J. Neurochem. *49*, 1222–1228.

Eagle, H. (1955). The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. J. Exp. Med. *102*, 37–48.

Ferkany, J. W., and Coyle, J. T. (1983). Kainic acid selectively stimulates the release of endogenous excitatory acidic amino acids. J. Pharmacol. Exp. Ther. *225*, 399–406.

Garthwaite, J., Garthwaite, G., and Hajos, F. (1986). Amino acid neurotoxicity: relationship to neuronal depolarization in rat cerebellar slices. Neuroscience *18*, 449–460.

Gazzola, G. C., Dall'Asta, V., Franchi-Gazzola, R., and White, M. F. (1981). Cluster tray method for rapid measurement of solute fluxes in adherent cultured cells. Anal. Biochem. *115*, 368–374.

Gjessing, L. R., Gjesdahl, P., and Sjaastad, O. (1972). The free amino acids in human cerebrospinal fluid. J. Neurochem. *19*, 1807–1808.

Halliwell, B. (1987). Oxidants and human disease: some new concepts. FASEB J. *1*, 358–364.

Huettner, J. E., and Baughman, R. W. (1986). Primary culture of identified neurons from the visual cortex of postnatal rats. J. Neurosci. *6*, 3044–3060.

Jahr, C. E., and Stevens, C. F. (1987). Glutamate activates multiple single channel conductances in hippocampal neurons. Nature *325*, 522–525.

Kessler, M., Baudry, M., and Lynch, G. (1987). Use of cystine to distinguish glutamate binding from glutamate sequestration. Neurosci. Lett. *81*, 221–226.

Koh, J., and Choi, D. W. (1988). Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. J. Neurosci. *8*, 2153–2163.

Lehmann, A., Isacsson, H., and Hamberger, A. (1983). Effects of in vivo administration of kainic acid on the extracellular amino acid pool in the rabbit hippocampus. J. Neurochem. *40*, 1314–1320.

Lehmann, J., Ferkany, J. W., Schaeffer, P., and Coyle, J. T. (1985). Dissociation between the excitatory and "excitotoxic" effects of quinolinic acid analogs on the striatal cholinergic interneuron. J. Pharmacol. Exp. Ther. *232*, 873–882.

Makowske, M., and Christensen, H. N. (1982). Contrasts in transport systems for anoic amino acids in hepatocytes and a hepatoma cell line HTC. J. Biol. Chem. *257*, 5663–5670.

Malouf, A. T., Schnaar, R. L., and Coyle, J. T. (1984). Characterization of a glutamic acid neurotransmitter binding site on neuroblastoma hybrid cells. J. Biol. Chem. *259*, 12756–12762.

May, P. C., and Gray, P. N. (1985). The mechanism of glutamate induced degeneration of cultured Huntington's disease and control fibroblasts. J. Neurol. Sci. *70*, 101–112.

Mayer, M. L. Westbrook, G. L., and Guthrie, P. B. (1984). Voltage dependent block by Mg^{++} of NMDA responses in spinal cord neu-

rons. Nature *309*, 261–263.

McGeer, E. G., McGeer, P. L., and Singh, K. (1978). Kainate-induced degeneration of neostriatal neurons: dependency on cortico-striatal tract. Brain Res. *139*, 381–383.

Munson, P. J., and Rodbard, D. (1980). Ligand: a versatile computerized approach for characterization of ligand binding systems. Anal. Biochem. *107*, 220–239.

Murphy, T. H., Malouf, A. T., Sastre, A. Schnaar, R. L., and Coyle, J. T. (1988a). Calcium dependent glutamate cytotoxicity in a neuronal cell line. Brain Res. *444*, 325–332.

Murphy, T. H., Schnaar, R. L., Coyle, J. T., and Sastre, A., (1988b). Glutamate cytotoxicity in a neuronal cell line is blocked by membrane depolarization. Brain Res. *460*, 155–160.

Murphy, T. H., Parikh, A., Schnaar, R. L., and Coyle, J. T. (1989). Arachidonic acid metabolism in glutamate neurotoxicity. Ann. NY Acad. Sci., in press.

Perry, T. L., Hansen, S., and Kennedy, J. (1975). CSF amino acids and plasma-CSF amino acid ratios in adults. J. Neurochem. *24*, 587–589.

Rehncrona, S., Folbergrova, J., Smith, D. S., and Siesjo, B. K. (1980). Influence of complete and pronounced incomplete cerebral ischemia and subsequent recirculation on cortical concentrations of oxidized and reduced glutathione in the rat. J. Neurochem. *34*, 477–486.

Roberts, P. J. (1974). Glutamate receptors in the rat CNS. Nature *252*, 399–401.

Saez, J. C., Kessler, J. A., Bennet, V. L., and Spray, D. C. (1987). Superoxide dismutase protects cultured neurons against death by starvation. Proc. Natl. Acad. Sci. USA *84*, 3056–3059.

Samuelsson, B., Dahlen, S., Lindgren, J., and Rouzer, C. A. (1987). Leukotrienes and lipoxins: structures biosynthesis, and biological effects. Science *237*, 1171–1176.

Schanne, F. A., Kane, A. B., Young, E. E., and Farber, J. C. (1979). Calcium dependence of toxic cell death: a common pathway. Science *206*, 700–702.

Schnaar, R. L., Weigel, P. H., Kuhlenschmidt, M. S., Lee, Y. C., and Roseman, S. (1978). Adhesion of chicken hepatocytes to polyacrylamide gels derivitized with N-acetylglucosamine. J. Biol. Chem. *253*, 7940–7951.

Simon, R. P., Swan, J. H., Griffiths, T., and Meldrum, B. S. (1984). Blockade of N-methyl-D-asparatate receptors may protect against ischemic damage in the brain. Science *226*, 850–852.

Smith, J. D., Freeman, G., Vogt, M., and Dulbecco, R. (1960). The nucleic acid of the polyoma virus. Virology *12*, 185–196.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. *150*, 76–85.

Tietze, F. (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and tissues. Anal. Biochem. *27*, 502–520.

Watkins, J., and Evans, R. H. (1981). Excitatory amino acids. Annu. Rev. Pharmacol. Toxicol. *21*, 165–204.

Zaczek, R., Balm, M., Arlis, S., Drucker, H., and Coyle, J. T. (1987a). Quisqualate-sensitive, chloride-dependent transport of glutamate into rat brain synaptosomes. J. Neurosci. Res. *18*, 425–431.

Zaczek, R., Arlis, S., Markl, A., Murphy, T., Drucker, H., and Coyle, J. T. (1987b). Characteristics of chloride dependent glutamate incorporation into brain membranes argue against a receptor binding site. Neuropharmacol. *26*, 281–287.